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Mutations in the Human Cytomegalovirus UL27 Gene That Confer Resistance to Maribavir

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Previous drug selection experiments resulted in the isolation of a human cytomegalovirus (CMV) UL97 phosphotransferase mutant resistant to the benzimidazole compound maribavir (1263W94), reflecting the anti-UL97 effect of this drug. Three other CMV strains were plaque purified during these experiments. These strains showed lower-grade resistance to maribavir than the UL97 mutant. Extensive DNA sequence analyses showed no changes from the baseline strain AD169 in UL97, the genes involved in DNA replication, and most structural proteins. However, changes were identified in UL27 where each strain contained a different mutation (R233S, W362R, or a combination of A406V and a stop at codon 415). The mutation at codon 415 is predicted to truncate the expressed UL27 protein by 193 codons (32% of UL27) with a loss of nuclear localization. The expression of full-length UL27 as a green fluorescent fusion protein in uninfected fibroblasts resulted in nuclear and nucleolar fluorescence, whereas cytoplasmic localization was observed when codons 1 to 415 were similarly expressed. Viable UL27 deletion mutants were created by recombination and showed slight growth attenuation and maribavir resistance in cell culture. Marker transfer experiments confirmed that UL27 mutations conferred maribavir resistance. The UL27 sequence was well conserved in a sample of 16 diverse clinical isolates. Mutation in UL27, a betaherpesvirus-specific early gene of unknown biological function, may adapt the virus for growth in the absence of UL97 activity.

Human cytomegalovirus (CMV) causes tissue-invasive diseases such as pneumonia, retinitis, and gastrointestinal disease in immunosuppressed patient populations. Currently available systemic therapy involves ganciclovir, foscarnet, and cidofovir, usually used in that order and sometimes used in combination. These drugs are deficient in several respects: they may have significant adverse effects (hematologic and renal), foscarnet and cidofovir require intravenous therapy, and there is a potential for cross-resistance resulting from viral DNA polymerase mutations (5) because all three drugs ultimately target the viral DNA polymerase. Among the more promising experimental anti-CMV agents are the benzimidazole ribosides. One subset of these drugs consists of D-ribose derivatives, which inhibit CMV by antagonizing the postsynthesis cleavage of concatemeric DNA to unit-length genomes and packaging into nucleocapsids (12). Another subset of benzimidazoles is exemplified by the L-ribose derivative maribavir (1263W94). The drug is a potent inhibitor of CMV and Epstein-Barr virus, including CMV strains resistant to currently available drugs (4, 9). Maribavir has good oral bioavailability, with few limiting side effects noted in a phase I clinical trial, which showed marked reduction in CMV shedding in semen at 4 weeks in human immunodeficiency virus-infected subjects (13). The mechanism of action of maribavir does not involve inhibition of the viral DNA polymerase, and laboratory-derived mutants resistant to maribavir did not have the UL56 or UL89 gene mutations of strains resistant to benzimidazole D-ribosides (4). A maribavir-resistant CMV strain was found to have a mutation at codon 397 of UL97, and enzyme kinetic studies showed that maribavir was a direct inhibitor of the CMV UL97 phosphotransferase (4). Three additional maribavir-resistant CMV strains derived in the course of this work did not contain mutations in the UL97 gene and are characterized in this report.

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MATERIALS AND METHODS

Antiviral compounds. Maribavir (1263W94) and its carbocyclic analog (2916W93) were synthesized in the Glaxo-Wellcome research laboratories (4). Compounds were solubilized in dimethyl sulfoxide (DMSO) at stock concentrations of 100 mM.

Cells, viruses, and clones. Human CMV strain AD169 was originally obtained from the American Type Culture Collection (Rockville, Md.), passaged locally, and plaque purified. A baseline stock was retained for sequence comparison with virus that was propagated under drug. Virus was grown in MRC5 human lung fibroblasts (BioWhittaker, Walkersville, Md.) or human foreskin fibroblasts (HFF). Sixteen clinical CMV isolates were selected for analysis of strain variation in UL27. They represented a diversity of genotypes or strain variants in other CMV gene regions. A strain AD169 derivative (RCd97-19) with most of the UL97 coding sequence deleted and replaced with a selectable marker was obtained from Mark Prichard (17). Plasmid vector pEFGP-N1, a green fluorescent protein (GFP) expression vector containing a cloning site at the N terminus for production of GFP fusion proteins, was obtained from BD-Clontech (Palo Alto, Calif.). Plasmid vector pGEM3z, containing T7 and SP6 promoters for the production of labeled RNA transcripts, was obtained from Promega Corp. (Madison, Wis.). Plasmid MP234, containing a GFP expression cassette (CMV immediate-early [IE] promoter, modified GFP, and a simian virus 40 polyadenylation site) was obtained from Mark Peeples (Rush-Presbyterian-St. Luke's Medical Center, Chicago, Ill.).

Antiviral susceptibility assays. Conventional plaque reduction assays gave variable results with maribavir, depending on cell culture conditions, and were not used in this study. During propagation under conditions of drug exposure, resistance was detected by a multicycle 96-well dot blot DNA hybridization assay

(4). MRC5 cells were infected with CMV at a multiplicity of infection (MOI) of ~0.01. Incubation was carried out at 37°C for 6 days, or approximately two rounds of viral replication. The amount of CMV DNA in each sample was then quantitated by radioactive DNA-DNA hybridization. The relative amounts detected with and without various concentrations of drug were used to screen for resistance (4). After resistant viruses were plaque purified, their resistance phenotype was determined by a multicycle yield reduction assay (5). Twenty-four-well monolayer HFF cultures that were confluent for 3 days were inoculated with CMV at an MOI of ~0.02 and cultured under exposure to eight maribavir concentrations ranging from 0 to 2 μM for 7 days. Culture supernatants were then removed, and their infectivity titers were assessed by inoculation onto fresh HFF monolayers and by immunoperoxidase staining the following day for CMV IE antigen, as previously described (5). The number of stained cell nuclei, which was enumerated by digital photomicroscopy and image analysis software (ImageJ; National Institutes of Health), corresponds to units of infectivity (5). The amount of reduction in infective virus yield at a single maribavir concentration of 1 μM was useful for screening purposes, and the drug concentration required to reduce the yield by 50% (50% inhibitory concentration [IC_{50}]), as determined by titration with drug concentrations above and below the IC_{50} value, was used for comparing maribavir resistance phenotypes. The IC_{50} values were an average of at least three determinations and are shown as a mean ± standard deviation (SD) value.

Growth curves were determined in single-cycle experiments (MOIs of 1.4 to 3) by inoculating and culturing in 12-well HFF monolayers and sampling 1 ml of culture supernatant daily for 4 days. DMSO was added to the sample to a 10% concentration, and the sample was frozen at −80C until all specimens were collected. The infectivity of the supernatants was then assayed in parallel by using similarly inoculated 24-well monolayers of HFF followed by IE antigen staining as described above.

DNA sequencing. Viral genomic DNA was extracted from infected fibroblast cultures with Tris-salt buffer containing sodium dodecyl sulfate and proteinase K. PCR products representing segments of the viral DNA were sequenced by using dye deoxy chain terminator chemistry on an ABI 377 (Applied Biosystems) instrument as previously described (5, 6). Hundreds of oligonucleotides were synthesized based on the AD169 sequence (GenBank accession number X17403), spaced at intervals of 250 to 500 nucleotides (nt) and covering large portions of the viral genome, for use as sequencing and PCR primers. Sequences were aligned with the published AD169 sequence (GenBank accession number X17403), and any sequence differences were compared with the sequence of the baseline AD169 stock used in this study.

RNA probes for blot hybridization. Segments of the AD169 reading frames UL26 (the entire open reading frame), UL27 (codons 1 to 415), UL28 (codons 156 to 340), and UL29 (codons 240 to 350), as well as the GFP coding sequence from plasmid MP234 (KpnI-PstI fragment, nt 1064 to 1349) were cloned into plasmid vector pGEM3z. Polymerases T7 or SP6 were used to generate negative-strand RNA probes for blot hybridization. The pGEM3z-derived clones were linearized with EcoRI or HindIII, and runoff transcripts were produced by using a Riboprobe kit (Promega) and digoxigenin-dUTP nucleotide mixture (DIG RNA labeling mix; Roche Molecular Biochemicals, Mannheim, Germany).

Northern blots. The baseline AD169 stock was used to infect 25 cm^2 of fibroblast monolayers at an MOI of 1 and then cultured with or without the addition of 20 μM ganciclovir. At 1 and 3 days after infection, the monolayers were lysed, and the total RNA was extracted by using a commercial kit (RNeasy, QIAGEN). One-third of the total RNA extract was denatured by using a glyoxal-DMSO loading buffer and was subjected to electrophoresis through a 1.1% agarose gel by using a Northern blotting kit (NorthernMax; Ambion). The separated RNA was blotted onto a positively charged membrane (BrightStar; Ambion) and hybridized according to the manufacturer's protocol (NorthernMax; Ambion) with digoxigenin-labeled UL27 RNA probe. After posthybridization washes, hybridized signal was developed by using anti-digoxigenin alkaline phosphatase conjugate and NBT-BCIP (nitrobule tetrazolium–5-bromo-4-chloro-3-indolylphosphate) substrate according to the manufacturer's protocol (DIG nucleic acid detection kit; Roche Molecular Biochemicals). Visualized bands were calibrated by using size markers (Millennium; Ambion) loaded at the time of RNA electrophoresis.

UL27 expression as GFP fusion proteins. The entire 608-codon *UL27* reading frame, or a truncated version containing only codons 1 to 415, was cloned in frame with the GFP coding sequence in the expression vector pEGFP-N1. The resulting plasmid (2 μg) was transfected into 25 cm^2 of fibroblast monolayers by using a lipid transfection reagent (Fugene; Roche Molecular Biochemicals). Green fluorescence was visualized with an inverted microscope with epifluorescence, and the cellular localization of the fluorescence was noted with each construct and with the unmodified pEFGP-N1 control. Photomicrographs were digitally recorded by a Nikon TE200 inverted fluorescent microscope equipped with the Applied Precision Deltavision deconvolution image restoration system (Oregon Health and Science University research core facility).

Deletion of UL27 in strain AD169 by insertional mutagenesis. The GFP expression cassette in plasmid MP234 was flanked on either side at its NsiI and MluI sites by the insertion of CMV strain AD169 sequences, yielding plasmid SC144 containing UL29 to UL28 on one side and UL27 (from codon 498 onward) to UL25 on the other side of the cassette. The orientation of the GFP cassette was opposite to the reading frames UL29 to UL26. PacI restriction sites were inserted at the upper and lower junctions of the GFP cassette. The resulting clone was digested with enzyme NotI and cotransfected with genomic AD169 DNA extracted as previously described (6). After viral cytopathology was observed, green fluorescence was used to identify the few cells containing replicating fluorescent recombinant virus. The recombinant virus (T2092) was isolated and purified by a combination of cell sorting (FACScan; Becton Dickinson) and plaque purification. The correct insertion of the GFP cassette and the deletion of codons 1 to 497 of the UL27 coding sequence were verified by PCR and Southern blotting. Southern blots were hybridized separately with the five digoxigenin-labeled probes defined above, representing UL26, UL27, UL28, UL29, and GFP. Hybridized probe was detected by using alkaline –phosphatase-labeled digoxigenin antibody as described for the Northern blots.

Transfer of UL27 mutations into strain AD169. The UL27 mutations identified in strains propagated under exposure to maribavir were individually transferred back into an AD169 background by digesting T2092 genomic DNA with PacI and cotransfection with cloned DNA representing nt 31236 to 36674 of the AD169 sequence and then modified to contain the desired UL27 mutation. A wild-type control sequence was also cloned, modified only to contain a unique SwaI site at codon 504 of UL27 that did not affect amino acid encoding. Recombinant virus was recognized by the absence of fluorescent cytopathology, and identity was further confirmed by the sequencing of the entire UL27 gene. The recombinant viruses were plaque purified and checked for maribavir sensitivity by yield reduction assays.

RESULTS

Isolation of maribavir resistant strains. Three populations (A, B, and C) of the baseline strain AD169 were serially passaged, mainly as infected cells, in increasing concentrations of the antiviral compound 2916W93 (4 to 80 μM) over the course of 12 months (Fig. 1). The carbocyclic analog of maribavir (2916W93) has about 20-fold less antiviral activity than maribavir, but no virus grew in cultures passaged in maribavir, and resistant virus that eventually grew under 2916W93 was cross-resistant to maribavir. The production of cell-free supernatant virus fell rapidly upon serial passage and was not detected after passage 5. At passage 9, some maribavir-resistant virus was detected by DNA hybridization in all three virus pools. Pool A was further propagated five more times under increasing concentrations of 2916W93 (up to 80 μM), and this resulted in the isolation of a highly maribavir-resistant supernatant virus, 2916rA, containing UL97 mutation L397R, which has already been reported (4). As a by-product of experiments attempting to transfer maribavir resistance from cosmid-cloned 2916rA DNA (4), plaque-purified strain rA5-13, isolated after several rounds of maribavir selection, was found to have low-grade maribavir resistance but no mutations in UL97. The B and C pools were propagated five more times under exposure to 10 μM maribavir, and resistant plaques were then identified that produced ~100 times more supernatant virus under drug exposure at 34°C than produced by strain AD169. At this point, two of five plaques in the B pool and four of six plaques in the C pool were resistant. One resistant plaque was selected from each pool and plaque purified twice more without drug. These viruses were designated rB2-2-2 and rC4-2-2. The three plaque-purified viruses (rA5-13, rB2-2-2, and rC4-2-2) were further characterized in this study. Susceptibility

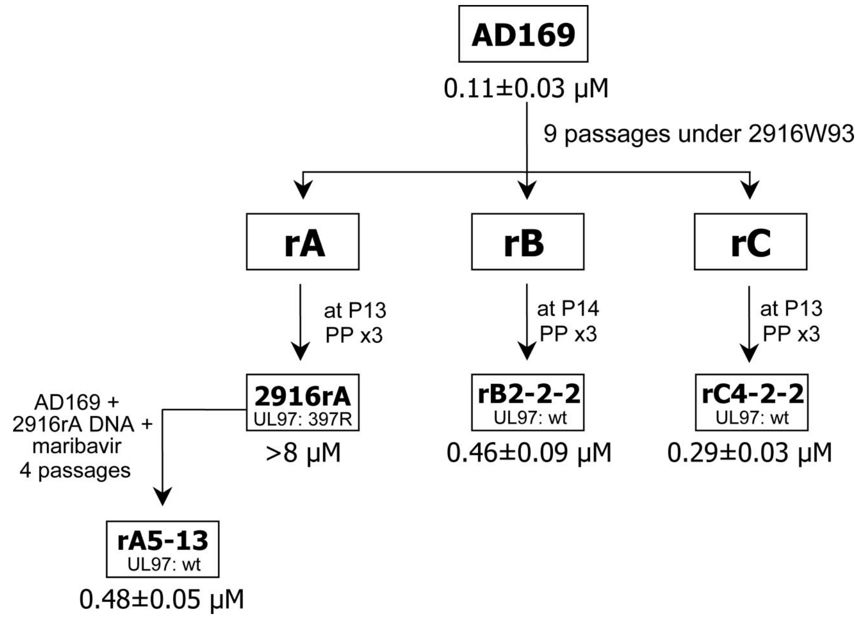

FIG. 1. Isolation of maribavir-resistant virus strains. After nine passages of strain AD169 under a maribavir carbocyclic analog (2916W93), three virus pools (rA, rB, and rC) were further propagated under conditions of drug exposure (rA under 2916W93 and the others under maribavir). At passage 13 (P13) or 14 (P14), the viruses were triply plaque purified (PP×3), resulting in the viruses shown, along with their UL97 genotype. The characterization of strain 2916rA has been published (4). DNA was extracted from 2916rA, cloned in cosmids, cotransfected with strain AD169 DNA, and selected four times under maribavir (4). One by-product of this process (rA5-13) was plaque purified. Sensitivity to maribavir, as determined by yield reduction assay, is shown as IC_{50} values (± SD, based on at least three determinations) under each strain.

testing (Fig. 1) showed that all three strains had low-grade maribavir resistance (three- to fourfold increase in IC_{50} over the AD169 control).

Sequence analysis of resistant strains. The complete UL97, DNA polymerase (UL54), and polymerase accessory protein (UL44) sequences were first determined for all three strains rA5-13, rB2-2-2, and rC4-2-2. All sequences were identical to the AD169 sequence. Subsequently, extensive portions of viral genomic sequence were determined for strains rB2-2-2 and rC4-2-2, covering the complete coding sequences of the reading frames UL23-35, UL42-57, UL69-105, UL112-3, and US22-24. These reading frames include almost all of the CMV genes known to be involved in viral DNA replication and assembly as well as most viral structural proteins. In several reading frames, coding sequences showed slight variation from published AD169 sequences. In most cases, the local baseline AD169 strain, rB2-2-2, and rC4-2-2 all showed the same variation. This was not considered significant for maribavir resistance. However, in reading frame UL27, the maribavir-resistant strains contained different mutations that were not present in the baseline strain AD169. Strain rB2-2-2 showed mutation A406V and a stop mutation at codon 415 that predicts truncation of the translated protein by approximately one-third. Strain rC4-2-2 showed the single point mutation W362R. After these mutations were detected, strain rA5-13 was also sequenced and found to have a different point mutation, R233S, in UL27. The previously published strain 2916rA (Fig. 1) (4) was sequenced and did not contain UL27 mutations.

UL27 sequence features and strain variation. The 1.8-kb UL27 reading frame predicts a translation product of 608 codons. We sequenced the gene in 16 clinical isolates selected for their diversity in other gene regions and found the peptide sequence to be 96% conserved, with no interstrain variation where the mutations were found in the maribavir-resistant viruses. Strain variations affecting amino acid encoding in the clinical isolates are shown in Table 1, where observed differences from the strain AD169 sequence (GenBank accession number X17403) are listed. Homology searches of public sequence databases revealed significant homology of UL27 only with the equivalent reading frames of a few betaherpesviruses; there was no homology with herpes simplex virus or any host protein. Even among betaherpesviruses, homology is limited. Among the sequenced CMVs, only chimpanzee CMV shows significant homology along most of the UL27 coding sequence. Shorter stretches of homology are observed with Tupaia, mouse, and rat CMV, and with human herpesviruses 6 and 7 (National Center for Biotechnology Information BLAST query). Recognized motifs in the amino acid sequence include four putative UL97 phosphorylation sites (SXXXXR) (2, 3), located at codons 173, 420, 492, and 555. The mutation in strain rA5-13 adds another such site (at codon 233), and the truncation in rB2-2-2 deletes three of them (beyond codon 415). There is a potential hydrophobic transmembrane domain at codons 386 to 405, just upstream of the predicted truncation site in strain rB2-2-2. Two potential bipartite nuclear localizing domain motifs in the terminal third of the gene (codons 448 to 465 and 496 to 512) are predicted to be lost in the truncated form of UL27 as expressed in rB2-2-2.

Kinetics of UL27 expression. Northern blot analyses were performed of total infected-cell RNA grown with and without ganciclovir to inhibit viral DNA synthesis and late gene expres-

TABLE 1. UL27 sequence variation in 16 clinical CMV isolates

Codon	Amino acid substitution	No. of isolates
11	Leu → Pro	3
12	Pro → Leu	1
22	Glu → Asp	1
84	Ala → Val	1
90	Lys → Arg	1
95	Pro → Ala	1
133	Leu → Ile	1
135	Val → Ile	3
221	Leu → Ile	1
288	Asp → Asn	1
289	Asn → Asp	14
294	Asp → Asn	2
297	His → Tyr	2
298	Asp → Gly	15
300	Asn → Gly	14
301	Asp → Asn	1
307	Pro → Leu	12
309	Ala → Val	1
310	Val → Ala	12
351	Asp → Asn	11
367	Ile → Val	15
465	Arg → Cys	1
557	His → Gln	7
558	Ala → Val	1
565	Ala → Thr	2

sion. Hybridization to the UL27 probe showed an initial predominant transcript size of 5 kb that was present with and without ganciclovir, indicating that UL27 is transcribed in the early phase of viral replication. This initial transcript size is about the same as previously reported with UL26 probes (18) and predicted to span the gene block UL29 to UL26 (reverse or right frame). Two days later, the dominant transcripts are shorter, ranging from 2.5 to 4.5 kb (Fig. 2). Evolution to these later transcripts is partially inhibited by ganciclovir.

Expression of UL27 as a fluorescent fusion protein. The complete UL27 coding sequence and a truncated form (retaining codons 1 to 415) as found in strain rB2-2-2 was inserted in frame upstream of a GFP sequence in expression vector pEGFP-N1. The complete UL97 sequence was likewise cloned in this vector as a control. Transfection of 2 μg of each vector DNA into fibroblasts and examination under epifluorescence showed different patterns of localization (Fig. 3). The fusion protein containing full-length UL27 showed nuclear and nucleolar localization, while the one containing the truncated version of UL27 showed cytoplasmic localization. UL97 showed nuclear localization without nucleolar localization.

UL27 deletion mutant. Codons 1 to 497 of UL27 were deleted after cotransfection of strain AD169 genomic DNA and plasmid SC144 containing a GFP expression cassette and flanked with UL29-28 and UL26-25 sequences. After purification through two cycles of fluorescence-activated cell sorting and three cycles of plaque purification from supernatant virus, the resulting CMV strain (T2092) showed 100% green fluorescent cytopathology. Evidence of the desired genetic construct included sequencing of the UL27 region showing the inserted GFP expression cassette, PCR with primers within and immediately outside the deleted segment showing the expected absence or presence of amplification products, and

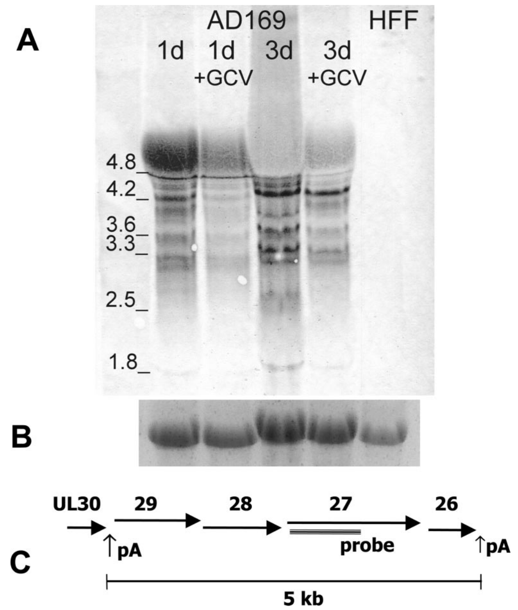

FIG. 2. Northern blot analysis of UL27 gene expression. (A) Total RNA extracts of infected cells at 1 and 3 days, cultured with and without added ganciclovir, were denatured and run on agarose gels, Northern blotted to nylon membrane, and then hybridized with digoxigenin-labeled UL27 probe. An uninfected-fibroblast total RNA extract was included as a control and showed no signal (right lane). RNA size markers (data not shown) were run in parallel. (B) Ethidium-stained 18S cellular RNA was photographed before blotting as an indicator of RNA content in each extract. (C) At early times postinfection, a 5-kb band predominated, corresponding in length to the distance between polyadenylation (pA) motifs upstream of UL29 and downstream of UL26. At late times this was replaced by various shorter transcripts, a process that is partially inhibited by ganciclovir. GCV, ganciclovir.

Southern blots with UL29, UL28, UL27, UL26, and GFP probes (Fig. 4). The fluorescent cytopathology of T2092 was compared with strain AD169 and the UL97 deletion mutant RCd97-19, and the appearance was much closer to the normal AD169 cytopathology than to that of RCd97-19, which exhibited an abnormally slow-developing cytopathology, with many granular nuclear inclusions forming in the infected cells as the infection progressed. Single-step growth curves were performed to assess the effect of the UL27 deletion in T2092. The results (Fig. 5) showed ~0.5 log attenuation at the end of a cycle of replication. In contrast, the virus RCd97-19 showed much greater attenuation at a comparable time point. The maribavir-resistant UL27 mutants rA5-13, rB2-2-2, and rC4-2-2 showed single-step growth curves that could not be reliably distinguished from the baseline strain AD169.

Marker transfer of UL27 mutations associated with maribavir resistance. Virus T2092 was designed to have its GFP cassette flanked with PacI restriction sites for subsequent removal and replacement with UL27 mutant sequences. There are no PacI sites in the CMV AD169 genome. After digestion of the T2092 genomic DNA with PacI and cotransfection with the UL27 region plasmid-cloned DNA containing UL27 mutations of interest, nonfluorescent recombinant viruses were recovered and plaque purified (Fig. 6). Sequencing analysis confirmed that strain T2174 contained UL27 mutations A406V and C415-stop as found in rB2-2-2, that strain T2175 contained

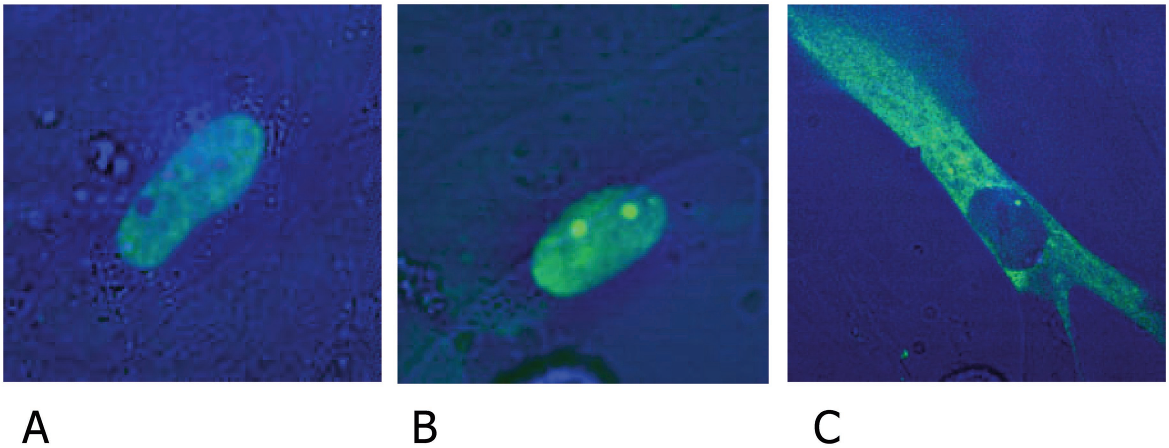

FIG. 3. Fibroblasts expressing GFP fusion proteins. UL97 (full-length) or UL27 (full-length or truncated) coding sequences were fused in frame with GFP coding sequences in vector pEGFP-N1 and transfected into fibroblasts. The UL97 fusion protein shows nuclear localization (A), the full-length UL27 fusion protein shows nuclear and nucleolar localization (B), and the fusion protein with UL27 truncated at codon 415 shows cytoplasmic localization with exclusion from the nucleus (C). Original magnification, ×60 (A and B) and ×40 (C).

UL27 mutation R233S as found in rA5-13, and that strain T2203 contained UL27 mutation W362R as found in rC4-2-2. No other mutations were found in UL27. A control wild-type recombinant virus T2194 was derived by replacing the fluorescent cassette in T2092 with the AD169 sequence, modified only to contain a unique SwaI site in UL27 that did not change the amino acid encoding. As a by-product of these experiments (Fig. 6), a recombinant nonfluorescent UL27 deletion mutant T2182 that was isolated and plaque purified was determined by PCR, sequencing, and Southern blotting (Fig. 4) to be the result of removing the fluorescent cassette from strain T2092 and religating the viral genome at the engineered PacI sites, without incorporation of the missing UL27 segment. Strain T2182, therefore, has UL27 codons 1 to 497 deleted and contains no foreign genetic elements to affect the interpretation of phenotype assays. Like the fluorescent T2092 UL27 deletion

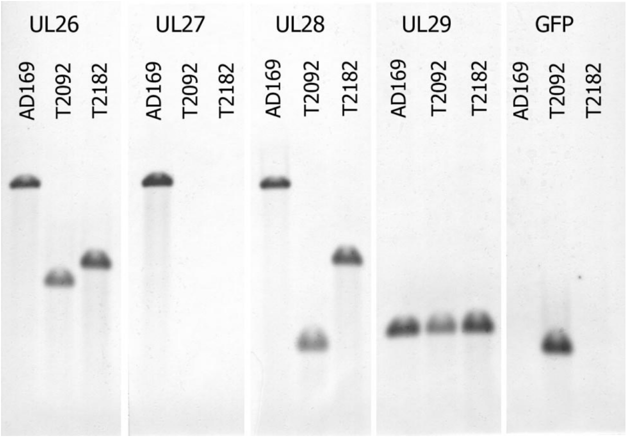

FIG. 4. Southern blot analysis of UL27 deletion mutants. Viral genomic DNA extracts were digested with enzyme PstI, electrophoresed, and blotted to nylon. Multiple replicates of the three extracts (AD169, T2092, and T2182) were run on the same gel and blotted to the same membrane and then cut into strips and hybridized separately with one of five different probes representing the sequences of the UL26 to UL29 genes and GFP. For wild-type AD169, two PstI genomic DNA fragments are detected, one with probes UL26, UL27, and UL28 and the other with probe UL29. T2092 and T2182, which have most of UL27 deleted, show an absence of hybridization with the UL27 (codons 1 to 415) probe. T2092 shows different fragment sizes with UL28 and UL26 probes because of two PstI sites in the GFP sequence inserted in place of UL27, whereas T2182 shows the same fragment with probes UL26 and UL28 because the GFP insert has been removed. The GFP probe hybridizes only to strain T2092 as expected, and neither strain T2092 nor T2182 shows the AD169 fragment size detected by probes UL26 to UL28, indicating absence of contamination with wild-type virus.

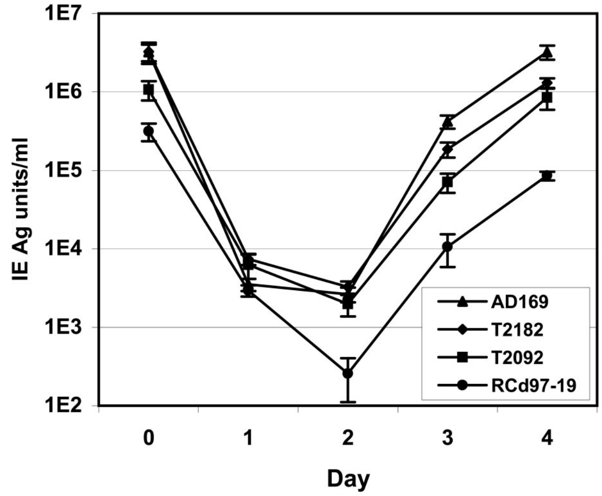

FIG. 5. Single-step growth curves of UL27 and UL97 deletion mutants. Extracellular virus concentrations were sampled daily after inoculation of various strains at an MOI of >1.4. Compared with wild-type strain AD169, the UL97 deletion mutant RCd97-19 (17) was attenuated by ~1.5 logs at 4 days. The UL27 deletion mutants T2092 and T2182 were slightly attenuated (~0.5 log at 4 days). The viral quantitations are averages of data from six experiments per virus, and the error bars show ± 1 SD. The day 0 quantitations are of the input virus.

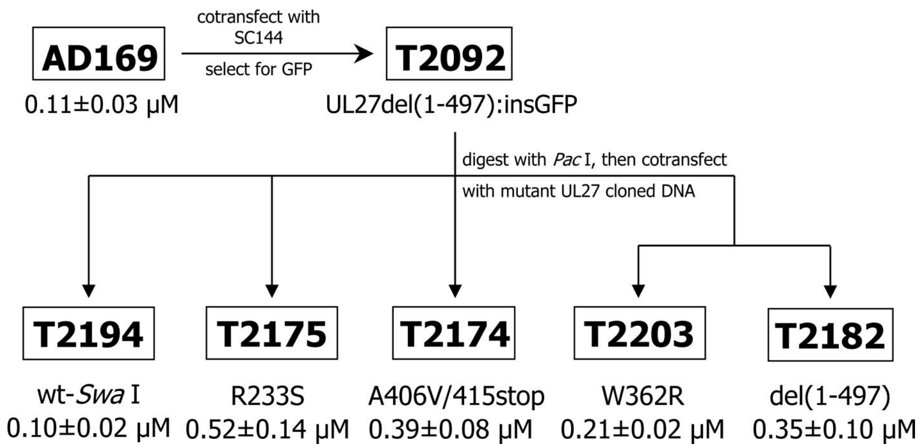

FIG. 6. Recombinant viruses containing specific UL27 mutations. Marker transfers were accomplished by digesting T2092 genomic DNA with PacI to remove the GFP coding sequence, leaving a gap at UL27. This was repaired by recombination following cotransfection with overlapping plasmids containing the desired UL27 sequence configurations. As a by-product of experiments used to produce T2203, a new strain T2182 was isolated in which no cotransfected UL27 DNA was incorporated, resulting in a nonfluorescent UL27 deletion mutant. Recombinant viruses were found to have the UL27 genotype and maribavir resistance phenotypes shown (IC_{50} ± SD values from at least three determinations are shown under the strain names).

mutant, strain T2182 was also modestly attenuated in cell culture when assessed by single-step growth curves (Fig. 5).

Yield reduction assays were performed with maribavir on all five viruses resulting from marker transfer (T2174, T2175, T2182, T219,4 and T2203). Strains T2174, T2175, and T2203 showed the same low-grade maribavir resistance phenotype (Fig. 6) as their respective UL27 mutants that were originally plaque purified after drug selection (rB2-2-2, rA5-13, and rC4-2-2). T2194 reverted to wild-type levels of maribavir susceptibility, and results on T2182 showed that deletion of UL27 also conferred a low-grade maribavir resistance similar to the other UL27 mutants.

DISCUSSION

Drug selection experiments with CMV laboratory strain AD169 resulted in the isolation of three maribavir-resistant strains containing different UL27 mutations, including point mutations and a premature stop codon predicting a truncation of the terminal third of the translated sequence. Marker transfer experiments confirmed that various mutations in UL27 (point mutation, truncation, and deletion) were capable of conferring maribavir resistance. This result contrasts with results for UL97, where truncations and deletions of more than a few codons have not been observed in connection with the isolation of drug-resistant mutants.

A previously reported CMV strain 2916rA (4) containing the mutation L397R in the UL97 protein showed a much higher level of maribavir resistance (Fig. 1), but mutation in UL97 appears to be less frequently selected in cell culture when compared to the multiple UL27 mutants identified here during the same series of experiments. An additional UL27 mutation (L355P) selected in cell culture has recently been linked to maribavir resistance as well (8). The UL27 gene appears to be more tolerant of mutation than the UL97 gene, at least in a laboratory strain propagated in cell culture. It is unknown at present if the initial selection of UL27 mutants with low-grade maribavir resistance facilitates the emergence of more highly resistant strains containing UL97 mutations.

The UL27 gene is transcribed starting in the early phase of viral replication, initially as part of a longer transcript and changing to shorter ones later in infection (Fig. 2). It is shown to be nonessential for viral replication since the recombinant viruses T2092 and T2182 lacking the first 497 codons of the gene are only slightly attenuated (Fig. 5). Yet the gene is well conserved among clinical isolates (Table 1) and may serve an important function in vivo. Of interest, the UL27 homolog of murine CMV, known as M27, was mutated in the course of random transposon mutagenesis (1), resulting in the truncation of M27 expression at codon 477 of the 682-codon gene. This M27 mutant was not appreciably growth impaired in cell culture but was attenuated in vivo, including in immunodeficient SCID mice (1), where mortality was prevented and much lower titers of virus were found in various tissues. This also supports a biologically important role for the UL27 gene, although there is very little knowledge about this gene and its role in different betaherpesviruses.

Maribavir is known to be a direct inhibitor of the UL97 phosphotransferase (4), and there is evidence that both maribavir-inhibited wild-type CMV and the UL97 deletion mutant RCd97-19 have defects in encapsidation or nuclear egress (10, 19). Encapsidation and nuclear egress involve the action of a number of viral gene products (15), including a kinase-mediated dissolution of the nuclear lamina during egress (16). Exactly how UL97 phosphotransferase or kinase activity facilitates viral replication is unclear, and it is likely that UL97 affects multiple steps in viral replication. The picture is complicated by the multiple potential substrates for UL97 (2, 3) and by overlap between the functions of cellular and viral kinases (7). For example, the CMV UL44 protein is a substrate for UL97 (11, 14), and absence of UL97 activity could affect the formation of replication complexes (14) prior to DNA replication. UL27 could be yet another substrate for UL97, given the presence of several proposed UL97 substrate motifs in UL27 (3) and the early expression and nuclear colocalization of both gene products UL27 and UL97. Phosphorylated UL27 could play a role in the nuclear egress of subviral particles, a process that appears to be defective in the absence of UL97 activity.

UL27 mutations probably adapt the virus to a lack of UL97 activity resulting from maribavir inhibition or from deletion of UL97, as in the mutant virus RCd97-19. Different mutations in UL27 may have different functional effects. The truncation of the terminal third of the protein with the loss of nuclear localizing signal motifs, as in rB2-2-2, probably alters the cellular localization of pUL27, as supported by observations with GFP fusion proteins (Fig. 3). The lack of phosphorylation of pUL27 may make its nuclear localization counterproductive, a concept that is consistent with the low-grade maribavir resistance detected in the recombinant virus T2182, which has most of UL27 deleted.

Since deletion of UL27 perceptibly attenuates viral growth, there may be a preferential selection of point mutations and partial truncations in UL27 after exposure to maribavir. Point mutations in UL27, as observed in strains rA5-13 or rC4-2-2, could make it more efficiently phosphorylated by a lower level of UL97 activity or by host kinases. Of interest is that the mutation R233S in strain rA5-13 introduces an additional serine residue that fits the recently proposed UL97 phosphorylation site motif (3). Further studies will be needed to address the relationship between UL27 and UL97.

The UL27 mutations studied here confer only low-grade maribavir resistance as measured by yield reduction assays. The clinical significance of this degree of resistance has not been determined. During clinical trials it will be necessary to monitor UL27 for mutations in any virus isolated after maribavir therapy and determine whether they are associated with persistent viral shedding or predispose the virus to the accumulation of additional mutations and higher-level resistance.

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